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ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF BACTERIA ON TWO SELECTED MEDICINAL PLANTS

Akala, A.O., Adegboyega, D.A., Olatunji, A.O., Kolawole, I.O., Edet, S.E, Oloyede, E.O and Akanni, F.O
Forestry Research Institute of Nigeria, Ibadan

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ABSTRACT

Bacterial isolates from Phyllanthus amarus and Azadiractha indica were characterized and identified as a preliminary step in determining an elimination treatment. The 22 bacteria were characterized using biochemical and morphological tests and subjected to sensitivity tests with four antibiotics. The isolates were compared with known organisms and assigned to genera according to similarities in characteristics. Seven isolates were analyzed by fatty acid analysis. Six were classified as Agrobacterium radiobacter; eight as Xanthomonas; one each as Pseudomonas fluorescens, Micrococcus spp., Corynebacterium spp., and Curtobacterium spp.; four could not be assigned to genera. Inhibition of growth of the bacteria by most antibiotics was best at pH 7.5.Minimal inhibitory concentration and minimal bactericidal concentrations of Gentamicin, Rifampicin, Streptomycin Sulfate, and Timentin varied with genotype.

Keywords: Elimination, Isolates, Bactericidal and Inhibitory.

1. INTRODUCTION

Phyllanthus amarus

Phyllanthus amarus is a plant of the family Euphorbiaceae and has about approximately 800 species which are found in tropical and subtropical countries of the world. Traditionally, *Phyllanthus amarus* herb has found its usefulness in the treatment of several health problems such as diarrhoea, dysentery, dropsy, jaundice, intermittent fevers, urinogenital disorders, scabies and wounds. Topically, it is used for several skin problems ranging from skin ulcers, sores, swelling and itchiness, wounds, bruises, scabies, ulcers and sores, edematous swellings, tubercular ulcers, ringworm, scabby and crusty lesions. Its effect in excretory system is due to its antiurolithic property and it is used in the treatment of kidney/gallstones, other kidney related problems, appendix inflammation and prostate problems (Khatoon *et al.,* 2004; Sen and Batra, 2013; Ushie *et al*., 2013). The secondary metabolites present in *P. amarus* are Alkaloids, Flavonoids, Hydrolysable Tannins (Ellagitannins), Major Lignans, Polyphenols, Riterpenes, Sterols and volatile oil. The main active constituents of *P. amarus* are Lignans (Phyllanthin, Hypophyllanthin, Nirurin Niranthin, Phyltetralin, Niranthine, Nirtetralin etc. (Morton, 1981; Chevallier, 2000; Srivastava *et al.,* 2008; Kassuya *et al.,* 2006; Huang *et al*., 2003; Maciel *et al.,* 2007; Singh *et al*., 2009), Flavonoids (Foo and Wong, 1992; Londhe *et al.,* 2008;), (Foo, 1995), Triterpenes (phyllanthenol, phyllanthenone, phytllantheol etc.) (Maciel *et al.,* 2007; Foo and Wong, 1992), Alkaloids (Houghton *et al.,* 1996; Kassuya *et al.,* 2006), Sterol (Amarosterol-A,

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Amarosterol-B etc.) (Ahmad and Alam , 2003) and volatile oil (Linalool, Phytol etc.) (Moronkola *et al*., 2009).
Hexane, methanol and water extracts of aerial parts of *P.amarus* were screened for antimicrobial activities *against Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus* and *Candida albicans* using the agar cup diffusion protocol. The aqueous and Methanolic extracts of *P. amarus* were active against all the test microorganisms. In another study, hexane, petroleum ether, chloroform, acetone and methanol extract of Phyllanthus leaves were tested for antibacterial activity against *Pseudomonasaeruginosa, Klebsiella pneumonia, Proteus mirabilis, Streptococcus faecalis, Enterobacter species, Serratia marcescens,Staphylococcus aureus* and *Escherichia coli* by agar well diffusion method. The results demonstrated methanol extract of *Phyllanthus amarus* for highest inhibitory activity against the above bacterial species (Saranraj and Sivasakthivelan, 2012)

The antidiabetic potential of *Phyllanthus amarus* investigated in an experiment model where fasted rats were made diabetic by single intraperitoneal injection of 120 mg/kg of Alloxan monohydrates and then two doses of the aqueous and hydroalcoholic extract of *Phyllanthus amarus* administered orally which were then compared with the normal control group that received distilled water only. After 15 days treatment the result demonstrates aqueous and hydroalcoholic extract of *Phyllanthus amarus* decrease the blood glucose level significantly. Serum analysis of the treated experimental animals showed an increase in insulin and reduction in the Malondialdehyde concentration, therefore demonstrated the potential antidiabetic property of aqueous and hydroalcoholic extract of *Phyllanthus amarus* (Evi and Degbeku, 2011).

Iranloye *et al* (2011), investigated the aqueous leaf extract of P. amarus for analgesic and anti-inflammatory activities using both thermal and chemical models of pain assessment in rats. The extract caused a significant ($p < 0.05$) dose related increased inhibition of the carrageenan-induced paw oedema in the rats. The inhibition produced by 200 mg/kg aqueous extract of P. amarus (70.20%) was significantly higher than that of the reference drug (acetylsalicylic acid). The extract produced a marked analgesic activity by inhibiting both early and late phases of pain stimulus in formalin induced paw licking rats and also a significant and dose related increase in inhibition of the mean tail immersion duration at varying water bath temperature (50, 55 and 60 $^{\circ}$C).

Azadirachta indica

Azadirachta indica is a fast growing, evergreen tree found commonly in India, Africa and America. It is a highly esteemed tree with several beneficial properties and applications, especially known for its incredible therapeutic and ethnomedicinal values for mankind. Moreover, it has antiseptic, antifungal, antibacterial, antipyretic, anti-malaria, anti-diabetic and anti-fertility properties among several other uses (Nok *et al*., 1993, Natarajan *et al*., 2003; Fredros*et al*., 2007; Mbaya *et al*., 2010).Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally variable with more than 140 compounds isolated from different parts of the tree (Subapriya and Nagini, 2005).

These compounds have been divided into two major classes: isoprenoids and others (Dev kumar and SukhDev, 1996). The Isoprenoids include Diterpenoids and Triterpenoids containing

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Protomeliacins, Limonoids, Azadirone and its derivatives, Gedunin and its derivatives, Vilasinin type of compounds and Csecomeliacins such as Nimbin, Salanin and Azadirachtin. The nonisoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as Flavonoids and their glycosides, dihydrochalcone, Coumarin and Tannins, Aliphatic compounds, etc. (Kraus, 1995; Dev kumar and SukhDev, 1996).

The susceptibility of the microorganisms to the extracts of Neem leaves was compared with certain specific antibiotics. The methanol extract of *Azadirachta indica* exhibited pronounced activity against *Bacillus subtilis*(Shravan *et al*., 2011). Neem oil preparations have been found effective against a wide spectrum of bacteria viz., *B. cerus, B. pumilus, S. aureus, M. tuberculosis, E. coli, P. vulgaris, S. typhi, K. pneumonae, S. dysenterae, Enterococcus faecalis, Streptococcus mutans, Streptococcus salivarius, Streptococcus mitis, Streptococcus sanguis* and even *Streptomycin* resistant strains (Sairam *et al*.,2000; Prashant *et al*., 2007; Mehrotra *et al*., 2010; Sarmiento *et al*.,2011;Maragathavalli *et al*., 2012; Vinothkumar*et al*., 2013; Rosaline *et al*., 2013). *Azadirachta indica* leaves possessed good antibacterial activity, confirming the great potential of bioactive compounds and is useful for rationalizing the use of this plant in primary health care (Saradhajyothi and Subbarao, 2011). Studies have also shown that Neem oil has been found to have definite antiplaque activity [Elavarasu *et al*., 2012]. Neem leaf extract can also inhibit the formation of biofilm in *Pseudomonas aeruginosa* [Harjai*et al*., 2013].

2. MATERIALS AND METHODS

Sample Collection and Processing

Fresh leaves of *Phyllantus amarus* and *Azadirachta indica* were collected and identified at the Forestry Research Institute of Nigeria, Oyo State, Nigeria. The leaves were washed properly to remove foreign matter and air dried. The leaves were then grinded to powder with a mechanical grinder, weighed and labeled. The powder was then subjected to various solvent extraction processes.

Source of Test Organisms

The bacteria were isolated from Crushed leaves of *A.indica* and *P.amarus* ,Soaked leaves of *A. indica* and *P.amarus* ,Buried leaves of *A.indica* and *P.amarus*. The isolates were subcultured to obtain pure cultures after which they were maintained on nutrient agar slants for further use.

Sterilization of Glassware and Media

All glassware used in this study were thoroughly washed with detergent, rinsed with water, air-dried and sterilized in hot air oven at 160^{o}C for two hours. Materials such as mouth of test tube, inoculating loop and inoculating needle were sterilized by flaming with a bunsen burner before and after inoculation to prevent contamination. All media used for isolation, cultivation and identification of isolates were sterilized by autoclaving at 121^{o}C for 15 minutes under pressure.

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Identification of Isolates

Identification of the isolates were done using Gram staining, morphological and biochemical characterisation of bacterial isolates was carried out(Olutiola *et al.*, 2000).The result of each test was recorded and the probable identity of the isolates was deduced with reference to Bergey's manual of Determinative Bacteriology (Bucchanam and Gibbons, 1974)

3. RESULT AND DISCUSSION

Table 1: Bacteria Isolates from *Phyllantus amarus.*

Bacteria Isolates	Isolate code
Staphylococcus sp	B1
Micrococcus sp	B2
Staphylococcus sp	B4
Staphylococcus sp	B6
Pseudomonas sp	B8
Micrococcus sp	B9
Bacillus sp	S1
Bacillus sp	S2
Bacillus sp	S3
Pseudomonas sp	S5
Pseudomonas sp	S6
Micrococcus sp	S8
Bacillus sp	G1
Bacillus sp	G2
Staphylococcus sp	G4
Corynebacteriumsp	G7
Corynebacteriumsp	G8

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Pseudomonas sp isolated from the soil and used as control was highly susceptible to both the aqueous and methanol extract of *Phyllanthus amarus* and also susceptible to the fractions of both extracts

The study also revealed that bacteria that had interacted with *Azadirachta indica* and *Phyllanthus amarus* were majorly resistant to the aqueous and methanol extracts of the plants. Also, the bacteria isolated from these plants also proved to be resistant to the ethyl acetate, chloroform and n-hexane fractions of the aqueous and methanol extracts of *Azadirachta indica* and *Phyllanthus amarus*, even though the bacteria used as control were all susceptible to the plant extracts at varying concentrations. This could be because the bacteria had developed resistance to the antimicrobials present in these plants during interaction and were no longer susceptible to these antimicrobials.

4. CONCLUSION

More than half a century has passed since the first antibiotics were introduced commercially but it did not take long for microbes to develop resistance to these antibiotics with widespread use of many antibacterial drugs providing ideal conditions for the spread of multi drug resistant organisms. Even though research is now focusing on the bioactive phytochemical constituents in medicinal plants as a source and template for the synthesis of new antimicrobial drugs, this study has revealed that bacteria have the tendency to develop resistance to plant antimicrobials after exposure to them. Further studies should be taken to determine the factors involved in the mechanisms of natural antimicrobial/multi-antimicrobial resistance in bacteria and ensure that reverting to the use of plants as new and alternative means of antimicrobial therapy does not eventually become a futile scientific endeavor.

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